Expression of Cholesterol Sulfotransferase (SULT2B1b) in Human Skin and Primary Cultures of Human Epidermal Keratinocytes

Yuko Higashi,* Hirotoshi Fuda,* Hidekatsu Yanai,* Young Lee,* Tomoko Fukushige,† Tamotsu Kanzaki† and Charles A. Strott*

*Section on Steroid Regulation, Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA; †Department of Dermatology, Faculty of Medicine, Kagoshima University, Sakuragaoka, Kagoshima, Japan

Cholesterol sulfate is a highly amphipathic molecule that is present in a relatively high concentration in the epidermis of human skin, particularly in the granular layer. The physiologic significance of this finding, however, is not well-understood. Therefore, we examined expression of the gene encoding for the enzyme that sulfonates cholesterol (SULT2B1b). Of the three enzymes known to sulfonate steroids/sterols, only the SULT2B1b isozyme was detected in cultures of normal human epidermal keratinocytes (NHEK) in response to Ca^{2+}-induced terminal differentiation as well as by normal human epidermal tissue. Immunocytochemical analysis of normal skin as well as specific skin disorders was carried out. In normal skin, the expression of SULT2B1b was localized to the granular layer of the epidermis similar to that of filaggrin, an acknowledged late marker of differentiation and in contrast to that of involucrin, an early marker of terminal differentiation, which was expressed throughout the suprabasal region. The confinement of SULT2B1b to the granular layer coincides with this being the area with the highest cholesterol sulfate content suggesting that the physiologic action of cholesterol sulfate is likely carried out in this region of the living epidermis. Additionally, 88% of cholesterol sulfate in NHEK was membrane-associated further suggesting a cellular location for cholesterol sulfate action.

Key words: cholesterol/epidermis/keratinocyte/sulfate/sulfotransferase
J Invest Dermatol 122:1207–1213, 2004

Cholesterol sulfate is a ubiquitous sulfolipid that has emerged as a multifaceted molecule with regulatory capabilities; it circulates at a relatively high concentration on the order of 150–300 μg per 100 mL (Strott and Higashi, 2003). Cholesterol sulfate is particularly prominent in the epidermis where it has been demonstrated to accumulate in the stratum granulosum (Lampe et al, 1983). Furthermore, epidermal keratinocytes accumulate cholesterol sulfate in culture during squamous differentiation (Jetten et al, 1989). Although cholesterol sulfoconjugating activity has been studied in human epidermal keratinocytes (Jetten et al, 1989), there are no reports on the expression of the genes involved in steroid/sterol sulfoconjugation in human skin or cultures of human epidermal cells.

The enzyme that produces cholesterol sulfate was recently identified in our laboratory as a member of the cytosolic sulfotransferase (SULT) superfamily (Javitt et al, 2001). The SULT superfamily is divided into five families, one of which (SULT2) is primarily engaged in the sulfoconjugation of neutral steroids and sterols (Nagata and Yamazoe, 2000). The human SULT2 family is further divided into two subfamilies, i.e., SULT2A1 and SULT2B1; furthermore, the SULT2B1 subfamily consists of two isoforms designated

SULT2B1a and SULT2B1b (Her et al, 1998). The prototypical steroid/sterol sulfotransferase, i.e., SULT2A1, commonly referred to dehydroepiandrosterone sulfotransferase, is capable of sulfonating a variety of steroids as well as bile acids (Radominska et al, 1990; Falany et al, 1994; Falany, 1997); however, it is only weakly active against cholesterol as a substrate (Fuda et al, 2002). Similarly, SULT2B1a, which preferentially sulfonates pregnenolone only weakly sulfonates cholesterol (Fuda et al, 2002). In contrast to SULT2A1 and SULT2B1a, however, SULT2B1b avidly sulfonates cholesterol and in this regard is more than an order of magnitude more active than the other SULT2 isozymes (Fuda et al, 2002).

Because of the importance of cholesterol sulfate in keratinocyte differentiation as well as development and maintenance of the epidermal barrier, we have examined the expression of SULT2B1b mRNA and protein in human skin as well as cultured human epidermal keratinocytes that had been subjected to Ca^{2+}-induced terminal differentiation. Additionally, cholesterol sulfotransferase activity and the subcellular localization of cholesterol sulfate during Ca^{2+}-induction of keratinocyte development were examined.

Results

Expression of SULT2 isozymes SULT2A1, SULT2B1a, and SULT2B1b mRNA and protein expression were
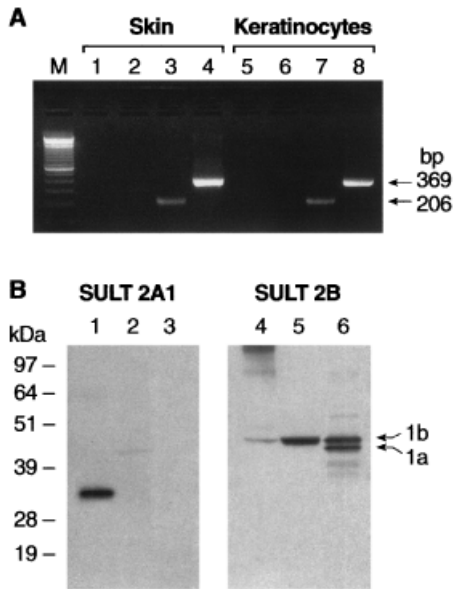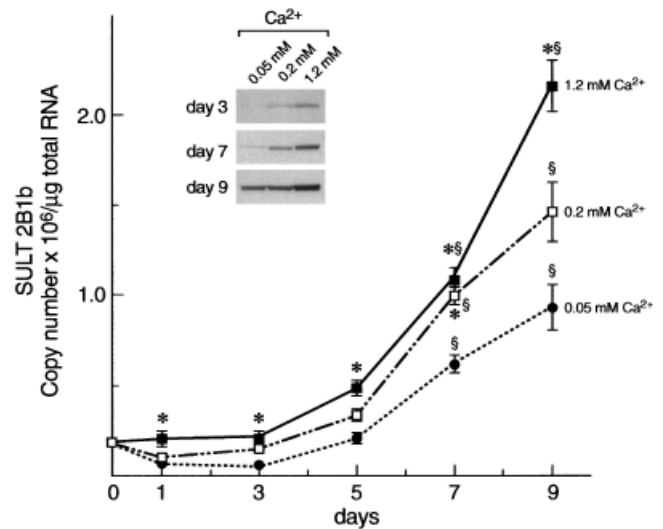

Figure 1
Expression of SULT2B1b in normal human skin and epidermal keratinocytes. (*A*) Reverse transcription-PCR analysis of SULT2A1 (*lanes 1,5*), SULT2B1a (*lanes 2,6*), SULT2B1b (*lanes 3, 7*) and β-actin (*lanes 4, 8*) of human skin (*lanes 1–4*) and NHEK after incubation with 1.2 mM Ca^{2+} for 9 days (*lanes 5–8*). (*B*) SULT2A1 and SULT2B1 isoforms were analyzed by immunoblotting. Extracts (30 μg) of normal human epidermis (*lanes 2, 4*) and NHEK that had been grown in 1.2 mM Ca^{2+} for 9 d (*lanes 3, 5*) were gel loaded. Positive controls were 15 ng of recombinant SULT2A1 (*lane 1*) and 70 ng each of SULT2B1a and SULT2B1b (*lane 6*).

Figure 2
Ca^{2+}-induction of SULT2B1b mRNA and protein expression in cultured normal human epidermal keratinocytes. Cells were treated with 0.05, 0.2, or 1.2 mM Ca^{2+}. At the indicated times, cell extracts and total RNA were prepared and analyzed by immunoblotting and real-time PCR. The inset shows expression of SULT2B1b protein after loading of 30 μg of cell extract. Real-time PCR values represent the mean ± SEM, n = 6. *p < 0.005 *vs* 0.05 mM Ca^{2+}-maintained cells using Scheffe's *F* test; §,time-dependent significance (Kruskal–Wallis ANOVA, p < 0.001) and p < 0.005 *versus* cells at day 0 by Scheffe's *F* test.

determined in normal human skin and primary cultures of NHEK. In both normal skin and NHEK, only SULT2B1b mRNA was expressed (Fig 1*A*). This was also the case for protein expression in the epidermis where only SULT2B1b protein was detected by western analysis (Fig 1*B*).

Calcium induction of SULT2B1b The effect of three Ca^{2+} concentrations, i.e., 0.05, 0.2, and 1.2 mM, on the quantitative expression of NHEK SULT2B1b mRNA and protein was examined. NHEK cells reached confluency in 4–5 d at 0.5 mM Ca^{2+} concentration and 7–9 d at either 0.2 mM or 1.2 mM Ca^{2+} concentration. At all three Ca^{2+} concentrations, the expression of SULT2B1b mRNA began to rise progressively in a concentration-dependent fashion by day 5 during the 9-d incubation period (Fig 2). The degree of enhancement was least at 0.05 mM Ca^{2+},

intermediate with 0.2 mM Ca^{2+} and maximal at 1.2 mM Ca^{2+}. The concentration-dependent inductionofSULT2B1b mRNA was mirrored by the concentration-dependent increase in SULT2B1b protein (Fig 2 *inset*).

Immunocytochemistry Staining for SULT2B1b in normal human skin was to all intents and purposes confined to the outer reaches of the living epidermis, i.e., the granular layer (Fig 3*A*). Faint staining noted in the dermis was indistinguishable from the light staining seen with pre-immune serum and is thus presumed to be non-specific (not shown). Whereas intense staining for involucrin was found throughout the suprabasal region (Fig 3*C*), filaggrin staining was confined to the granular layer of the living epidermis (Fig 3*B*). The staining pattern for SULT2B1b, however, did differ from filaggrin in that staining for the latter protein was also present in the stratum corneum (Fig 3*B*) in contrast to SULT2B1b, which appeared to be absent from the non-viable region of the epidermis (Fig 3*A*).
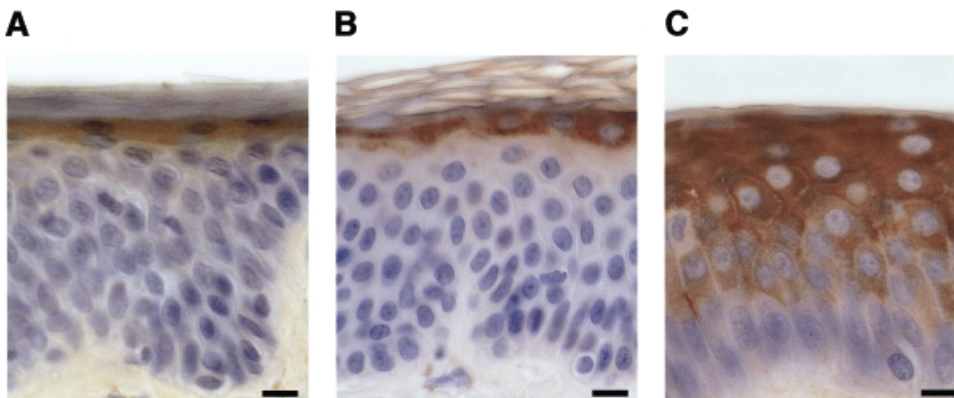
Figure 3
Immunohistochemical detection of SULT2B1b and barrier proteins in normal human skin. Tissues were stained with antibody to SULT2B1 (*A*), filaggrin (*B*) or involucrin (*C*). *Scale bar* = 10 μm.
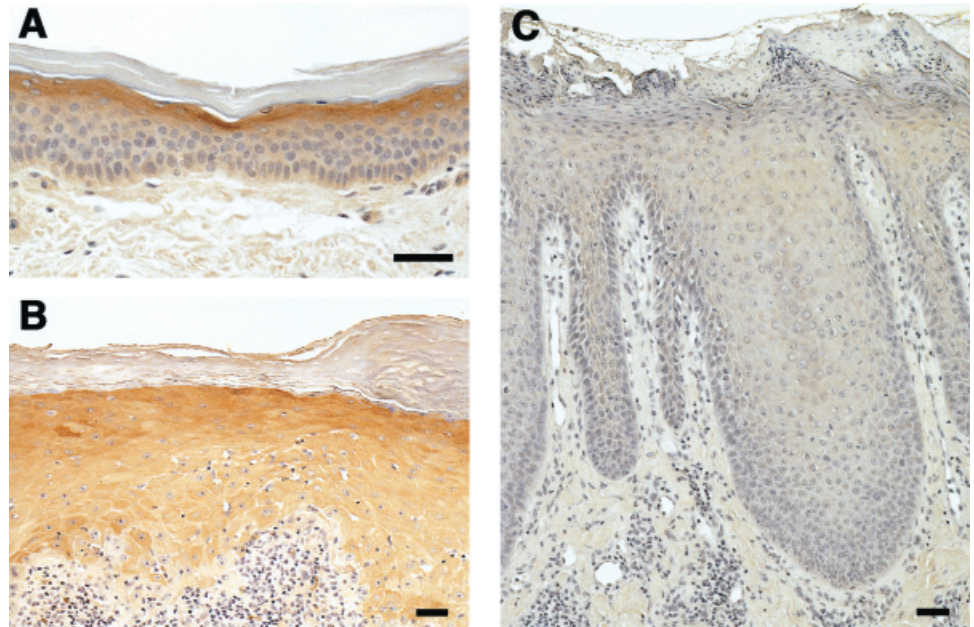
Figure 4
Immunohistochemical detection of SULT2B1b in disorders of human skin. Tissues were stained with antibody to SULT2B1 in recessive X-linked ichthyosis (*A*), lichen planus (*B*), and psoriasis vulgaris (*C*). *Scale bar* = 40 μm.

Additionally, epidermal expression of SULT2B1b was examined in three pathologic conditions. In recessive X-linked ichthyosis, SULT 2B1b expression was confined to the granular layer (Fig 4*A*) in a pattern that is similar to normal skin (Fig 3*A*). In lichen planus, the staining pattern for SULT2B1b was quite striking in that it revealed strong expression throughout the epidermis with the exception of the stratum corneum (Fig 4*B*). On the other hand, SULT 2B1b expression in a psoriatic plaque was essentially undetectable and was clearly absent in nucleated corneocytes (Fig 4*C*).

Cholesterol sulfotransferase activity Cholesterol sulfotransferase activity exhibited by cultured NHEK grown in either 0.05 or 1.2 mM Ca^{2+} was first determined at day 3 of incubation, and whereas the subsequent increase in activity was modest for cells maintained in 0.05 mM Ca^{2+}, the increase in activity was much greater for NHEK maintained in 1.2 mM Ca^{2+} (Fig 5). It should be noted that there is no 0 d value for absolute comparison as the cell count is too low to obtain enough protein for analysis. Nonetheless, NHEK exposed to 1.2 mM Ca^{2+} exhibited a striking increase in cholesterol sulfotransferase activity by day 9 as compared to the activity demonstrated by cells maintained in medium containing 0.05 mM Ca^{2+} (Fig 5). Importantly, these results mirror the effect of the Ca^{2+} concentration on the levels of SULT2B1b expression in cultured NHEK (Fig 2 *inset*).

Cellular localization of cholesterol sulfate Cultured NHEK were examined at day 9 during incubation at both low and high Ca^{2+} concentrations. In a preliminary experiment, we determined by RT-PCR that NHEK express the enzyme that produces 3′-phosphoadenosine 5′-phosphosulfate (PAPS) the universal sulfonate donor molecule required for all sulfotransferase reactions (data not presented). Since PAPS is produced from inorganic sulfate and ATP in a two-step process (Lyle *et al*, 1994), we incubated cells with $^{35}SO_4$ of known specific activity for incorporation into ^{35}S-labeled PAPS. The mass of cholesterol sulfate

was then determined from the incorporation of $^{35}SO_4$ into cholesterol sulfate. As expected, NHEK produced a significantly greater amount of cholesterol sulfate when maintained at the higher Ca^{2+} concentration (Fig 6). Interestingly, at both low and high Ca^{2+} concentrations ∼ 90% of the cholesterol sulfate was located in the membrane fraction, whereas only about 10% was in the soluble fraction (Fig 6).

Nota bene In arriving at an estimate for cholesterol sulfate at least two considerations had to be taken into account. First, since the culture medium contains a total of 3.653 μM sulfur in the form of SO_4 as noted previously, a correction was made in the $^{35}SO_4$ specific activity to reflect this dilution factor. Second, the ^{35}S-labeled PAPS produced
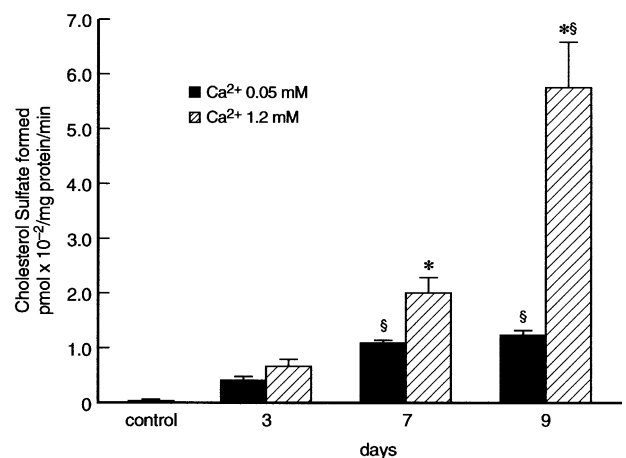

Figure 5
Cholesterol sulfotransferase activity in cultures of normal human epidermal keratinocytes. Cells were treated with either 0.05 or 1.2 mM Ca^{2+}. At the designated times, cells were detached, cytosol prepared and assayed for enzymatic activity. Control indicates activity in the absence of cytosol protein. Column heights represent mean values (n = 4); error bars are indicated. *p < 0.05 *vs* 0.05 mM Ca^{2+}-maintained cells using Scheffe's *F* test. §,Time-dependent significance (Kruskal–Wallis ANOVA, p < 0.005) and p < 0.005 *versus* cells at day 3 by Scheffe's *F* test.
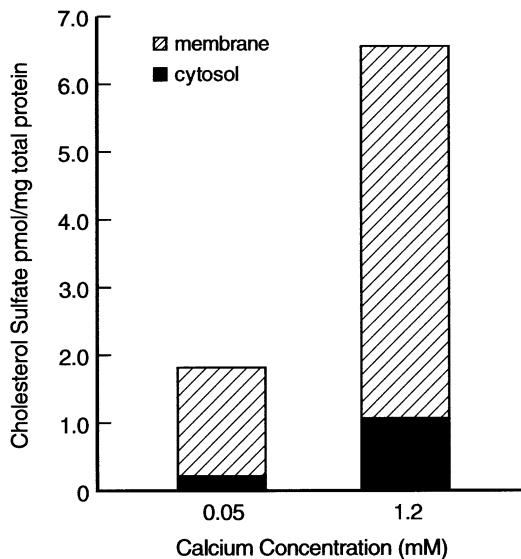
Figure 6
Subcellular localization of cholesterol sulfate in culture of normal human epidermal keratinocytes. Cells were cultured for 9 d in medium containing either 0.05 or 1.2 mM Ca^{2+} and 20 μCi per mL $Na_2^{35}SO_4$. Membrane and cytosol fractions were prepared and assayed for cholesterol sulfate. The results are representative of two independent experiments.

from $^{35}SO_4$ will also be diluted by the amount of endogenous unlabeled PAPS that is present; however, since the quantity of the latter is unknown a correction in the final specific activity in this case was not possible, and as a result the cholesterol sulfate levels reported are most likely an underestimation. Nevertheless, since the aim of this experiment was not to establish absolute values for cholesterol sulfate but rather to simply compare low and high calcium effects on cholesterol sulfate levels as well as the partitioning of cholesterol sulfate between membrane and cytosolic fractions, the conclusions drawn are considered valid.

Discussion

The end product of epidermal keratinocyte differentiation is development of an effective physical barrier of cornified squamous cells, i.e., the stratum corneum (Fuchs, 1990). The water impermeable barrier is composed of a layer of flattened anucleated corneocytes mortared together by lipid lamellae atop a layer of cross-linked cell envelope proteins anchored to a keratin filament–matrix complex (Nemes and Steinert, 1999). Structural defects in any of the components of this covering may lead to clinically significant disease resulting from impairment in barrier function (Roop, 1995). Notably, cholesterol sulfate has been found to be a significant component of stratum corneum lipids (Gray and Yardley, 1975; Long *et al*, 1985; Downing *et al*, 1987; Bouwstra *et al*, 2000), and evidence suggests that this highly amphipathic molecule plays a role in keratinocyte differentiation and development of the barrier (Strott and Higashi, 2003).

Interest in cholesterol sulfate as an important epidermal factor stems from the discovery of high levels of this sulfolipid in the epidermis of patients afflicted with the

hyperkeratotic scaling disorder known as recessive X-linked ichthyosis (Williams and Elias, 1981), an abnormality that is the consequence of a steroid sulfatase deficiency (Ballabio and Shapiro, 1995). Subsequently, literature expounding on various aspects of the significance of cholesterol sulfate in epidermal tissue appeared, and although a definitive function(s) for cholesterol sulfate in skin is(are) not clearly understood, several significant actions have been ascribed to it. For instance, cholesterol sulfate activates multiple protein kinase C isozymes (Denning *et al*, 1995), is a potent inhibitor of cholesterol synthesis (Williams *et al*, 1985), is able to induce transcription of the gene for transglutaminase I, an essential cross-linking enzyme involved in barrier formation (Kawabe *et al*, 1998), and is capable of regulating transcription of the gene for involucrin, a major cross-linked protein constituent of the insoluble cornified cell envelope (Hanley *et al*, 2001).

Consistent with the involvement of cholesterol sulfate in keratinocyte development was the finding of a progressive expression of SULT2B1b mRNA in primary cultures of NHEK during Ca^{2+}-induction of terminal differentiation along with a parallel increase in SULT2B1b protein and cholesterol sulfotransferase activity. It was also found that an increase in the level of involucrin mRNA followed a similar time-course as SULT2B1b induction (data not presented).

Of the three known steroid/sterol sulfotransferase isozymes, only SULT2B1b was expressed in skin and NHEK. This would be the expected result if the sole purpose of SULT2 expression in skin is the production of cholesterol sulfate since SULT2B1b, in contrast to the other twoSULT2 isozymes, functions as a selective cholesterol sulfotransferase (Fuda *et al*, 2002). It is of interest that the expression of SULT2B1b in skin is localized to the granular layer of the living epidermis similar to filaggrin, which is a late marker of keratinocyte differentiation (Fuchs, 1990), suggesting that SULT2B1b is also a late marker of differentiation. In contrast, involucrin, which is an early marker of keratinocyte differentiation (Fuchs, 1990), is strongly expressed in the immediate suprabasal spinous layer of the epidermis. It is noteworthy that the presence of SULT2B1b in the granular layer is consistent with this layer containing the highest concentration of epidermal cholesterol sulfate (Lampe *et al*, 1983; Elias *et al*, 1984).

Immunohistochemical analysis of SULT2B1b in disorders involving the integument revealed striking differences in staining patterns. For instance, in psoriasis vulgaris, SULT2B1b expression was essentially undetectable. Previous investigations revealed that whereas involucrin is expressed in the psoriatic epidermis, late differentiation markers such as profilaggrin and loricrin are greatly diminished or absent (Ishida-Yamamoto *et al*, 2000). The hallmark of psoriasis vulgaris is the absence of a granular layer and the presence of nucleated corneocytes. Cell envelopes form prematurely and are persistently involucrin-positive suggesting that psoriatic cell envelopes remain in their premature stage without further maturation (Ishida-Yamamoto *et al*, 2000). Thus, in psoriasis vulgaris, the apparent lack of normal cellular progression with formation of a granular layer would seem to explain the lack of expression of late markers of differentiation such as

profilaggrin, loricrin, and SULT2B1b. In contrast to psoriasis, expression of SULT 2B1b in lichen planus is observed throughout the epidermis with the exception of the stratum corneum consistent with this disorder being characterized as one with a thickened granular layer (Kanitakis *et al*, 1988).

An interesting finding was that almost 90% of the cholesterol sulfate formed during Ca^2-induction was found in the cellular membrane fraction with only 10% being present in the soluble cell fraction. Cholesterol sulfate localization to NHEK cellular membranes is consistent with a previous report noting that cholesterol sulfate was present in stratum corneum membranes (Elias *et al*, 1984). This finding along with previous reports of cholesterol sulfate localization to membranes of erythrocytes (Bleau *et al*, 1972; Bleau *et al*, 1974; Bleau *et al*, 1975) and spermatozoa (Langlais *et al*, 1981; Roberts, 1987) suggests that cellular membrane localization of cholesterol sulfate might be a general phenomenon and further suggests that this is an important determinant of its physiologic effect.

The localization of cholesterol sulfotransferase to the granular layer of the epidermis just beneath the stratum corneum along with the knowledge that this region of the epidermis contains the highest content of cholesterol sulfate strongly suggests that the principal function of this sulfolipid is carried out primarily in the region of the granular-stratum corneum junction. The epidermis is a perpetually renewing tissue whereby keratinocytes arise from stem cells in the basal layer, move through a series of cellular differentiation events until as dead squames they are finally sloughed off from the outer stratum corneum (Roop, 1995). It was previously suggested that cholesterol sulfate might play a significant role in the proper functioning of the stratum corneum and the normal sloughing of dead cells in a process termed desquamation (Long *et al*, 1985). It has been demonstrated that cholesterol sulfate is able to retard desquamation by inhibiting serine proteases in the stratum corneum that are responsible for the degradation of cell adhesion structures known as desmosomes (Sato *et al*, 1998). Additionally, cholesterol sulfate can influence cell cohesiveness by affecting the stability of corneocyte lipid bilayers (Bouwstra *et al*, 1999; Bouwstra *et al*, 2000). The inhibiting influence of cholesterol sulfate on normal desquamation, which presumably occurs maximally in the lower stratum corneum would subsequently be removed by the presence of a cholesterol sulfate sulfohydrolase in the outer stratum corneum thus allowing normal desquamation to occur (Ballabio and Shapiro, 1995). This hypothesis is supported by the finding of markedly elevated cholesterol sulfate in the stratum corneum of patients with X-linked recessive ichthyosis (Williams and Elias, 1981), a scaling disorder which is a form of abnormal desquamation resulting from a deficiency in stratum corneum steroid sulfatase activity (Elias *et al*, 1984). This hypothesis is further supported by the observation that increased thickness and scaling of the stratum corneum can be induced by the topical application of cholesterol sulfate, which is taken up and retained by the stratum corneum (Elias *et al*, 1984; Maloney *et al*, 1984). These interesting observations notwithstanding, however, the precise role of cholesterol sulfate in the epidermis remains to be firmly established.

Materials and Methods

Chemicals Cholesterol sulfate, adenosine 3′-phosphate 5′-phosphosulfate (purity > 80% by HPLC), calf collagen III, and 2-hydroxypropyl-β-cyclodextrin were purchased from Sigma (St Louis, Missouri). Organic solvents were purchased from Mallinckrodt Baker (Paris, Kentucky). Oligonucleotides were obtained from Gene Probe Technologies (Gaithersburg, Maryland). ^{3}H-Cholesterol (60 Ci per mmol) and $Na_2^{35}SO_4$ (1494 Ci per mmol) were purchased from Perkin-Elmer Life Sciences (Boston, Massachusetts). Silica gel TLC plates were obtained from Analtech (Newark, Massachusetts), and proteinase inhibitor cocktail Set V was obtained from Calbiochem (San Diego, California).

Cell culture Normal human epidermal keratinocytes (lot no. 15073) (obtained under informed consent and in adherence with the Helsinki principles) (NHEK) obtained from Cambrex (East Rutherford, New Jersey) were seeded in dishes that had been collagen-coated by incubation in a solution consisting of 0.1 mg per mL calf collagen III and 0.1 M acetic acid for 2 h at room temperature then washed with phosphate-buffered saline (PBS). NHEK were maintained in KGM-2 medium (Cambrex) containing 0.05 mM calcium and supplements (bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone, transferrin, epinephrine, gentamicin sulfate, and amphotericin-B). KGM-2 medium contains a total of 3.653 μM sulfur (2.743 μM $FeSO_4$, 0.0169 μM $CuSO_4$, 0.8916 μM $ZnSO_4$ and 0.001124 μM $MnSO_4$). NHEK on the third passage were used for all experiments. Cells (2.5×10^5) were seeded on 10 cm plates and after 24 h the cultures were fed fresh medium containing varying concentrations of calcium.

RT-PCR Total RNA was extracted from keratinocytes using Absolutely RNA RT-PCR Miniprep Kit according to the manufacturer's instructions (Stratagene, La Jolla, California). The RNA samples were treated with DNase supplied with the kit. Reverse transcription was performed using ThermoScript RT-PCR system according to the manufacture's instructions (Invitrogen, Carlsbad, California). Briefly, utilizing 1 μg of either human skin total RNA (Stratagene, lot no.0120484) or NHEK as a template first strand cDNAs were made using 25 pmol oligo(dT)20 and 25 ng of random hexamer primers in a 20 μL reaction volume. Following heat denature at 65°C for 5 min, reverse transcription was carried out at 25°C for 10 min and then 60°C for 50 min. Two microliter aliquots of cDNA were used as templates. Primers used were 5′-C(nt295)ACCTCCCCATCCAGTTATTCC(nt316)-3′ (sense) and 5′-G(nt581)TTCTTCCTGTGTCCTGTTTCAGC(nt558)-3′ (antisense) for SULT2A1, 5′-T(nt269)CACCACTTTACAGAAGAGGGACTG(nt293)-3′ (sense) and 5′-G(nt567)ATCTCGATCATCCAGGTCGTG(nt546)-3′ (antisense) for SULT 2B1a, 5′-G(nt112)GCTTGTGGGACACCTATGAAG(nt133)-3′ (sense) and 5′-A(nt317)TCTCGATCATCCAGGTCGTGC(nt296)-3′ (antisense) for SULT2B1b and 5′-C(nt261)TGGCACCACACCTTCTACAATG(nt283)-3′ (sense) and 5′-A(nt651)ATGTCACGCACGATTTCCCGC(nt630)-3′ (antisense) for β-actin. Expected sizes of SULT2A1, SULT2B1a, SULT2B1b, and β-actin are, respectively, 286, 299, 206, and 369 bp. PCR conditions were: denaturing at 95°C for 5 min, followed by 35 cycles of denaturing at 94°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s.

Real-time PCR Quantification of SULT2B1b mRNA was performed using a fluorescence temperature cycler (LightCycler, Roche Molecular Biochemicals, Indianapolis, Indiana) and SYBR Green I as a double-strand DNA-specific binding dye according to the manufacture's instructions. Real-time PCR was performed according to a previously published procedure using the same primers that were used for RT-PCR (Shimizu *et al*, 2003).

Preparation of recombinant proteins Recombinant SULT2 subtypes were prepared and purified using the glutathione S-transferase gene fusion system (Amersham Biosciences, Piscataway, New Jersey) according to a previously reported procedure (Fuda *et al*, 2002).

Antibody preparation Purified recombinant SULT2A1 and SULT2B1b were used for immunization (Covance, Denver, Pennsylvania). Antibodies to the SULT2 isozymes were produced in New Zealand white rabbits by a modification of the multiple intradermal protocol (Vaitukaitis, 1981), and immune sera were validated by western blot analysis using recombinant proteins. It should be noted that because of the high homology of the SULT2B1 isoforms (95% identical) the antiserum to SULT2B1b completely cross-reacts with SULT2B1a. Nonetheless, the two SULT2B1 isoforms can easily be distinguished on the basis of size.

Western blotting Skin tissue (lot no. 0041651) obtained from the National Resource Center (Philadelphia, Pennsylvania) was incubated in PBS for 3 min at 50°C and the epidermis separated from the dermis by gentle dissection. The epidermis was immediately homogenized in a solution consisting of 0.25 M sucrose, 0.05 M Tris (pH 7.2), 0.001M EDTA, and proteinase inhibitor cocktail using a polytron homogenizer. Homogenates were centrifuged at $1000 \times g$ for 10 min and the supernatants decanted for analysis.

Cultured NHEK were washed with ice-cold PBS, scraped into PBS containing proteinase inhibitor cocktail and sonicated on ice for 10 s 3 ×. Samples were electrophoresed on 10% Bis–Tris Gels (Invitrogen) and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, Maryland). Membranes were soaked in a solution of 5% dry milk (Bio-Rad, Hercules, California) in Tris-buffered saline (TBS) containing 0.05% Tween 20 for 30 min with gentle shaking, after which they were exposed to either SULT2B1 antibody (1:1000) or SULT2A1 antibody (1:32000) for 1 h. Membranes were washed 3 × with TBS containing 0.05% Tween 20 and incubated with goat anti-rabbit antibody (1:55,000, KPL, Gaithersburg, Maryland) for 30 min. Finally, membranes were washed 3 × in TBS containing 0.05% Tween 20, and signals were detected with LumiGLO (KPL) using the manufacturer's protocol prior to exposing the membranes to Scientific Imaging Film (Kodak, Rochester, New York).

Cholesterol sulfate and cholesterol sulfotransferase activity In experiments involving assays of either cholesterol sulfate or cholesterol sulfotransferase activity, NHEK were incubated with either 0.5 or 1.2 mM Ca^{2+} for up to 9 d. Additionally, in studies involving the assay of cholesterol sulfate, cells were incubated with 20 μCi per ml $Na_2^{35}SO_4$. At the end of each experiment, cells were washed 2 × with PBS, detached by scraping and homogenized with 10 strokes of a Dounce homogenizer (KONTES, Vineland, NJ). Nuclei and unbroken cells were pelleted by centrifugation at $1000 \times g$ for 10 min and the decanted supernatants centrifuged at $100,000 \times g$ for 1 h. High-speed pellets were washed 2 × with PBS and used as membrane fractions, whereas the high-speed supernatants constituted cytosolic fractions.

Cholesterol sulfotransferase activity was determined using ^{3}H-cholesterol according to a previously published method (Fuda *et al*, 2002). Samples (50 μL) consisted of 25-μg cytosolic protein in 0.1 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 0.2 mM 2-hydroxypropyl-β-cyclodextrin and 4% ethanol (vol/vol). Reactions were carried out at 37°C for 1 h, stopped by precipitation with 1 mL of ethanol for 30 min on ice and centrifuged. Supernatants were reduced by evaporation, 40 μL of cholesterol sulfate in methanol (0.25 mg per mL) were added as a carrier and the samples applied to TLC plates that were developed in chloroform/methanol/acetone/acetic acid/water (44:10:20:6:5). Following chromatography, TLC plates were dried and exposed to I_2 vapor to visualize the cholesterol sulfate spots, which were excised and the radioactivity determined by liquid scintillation spectrometry.

In the assay of cholesterol sulfate, samples were also subjected to ethanol precipitation and processed as above.

Immunocytochemistry Tissue samples used in these studies were obtained under a protocol approved by the Kagoshima University Faculty of Medicine Human Research Committee. Skin biopsies were obtained from healthy volunteers, as well as patients afflicted with psoriasis vulgaris, lichen planus, and recessive X-linked ichthyosis. Histochemical analysis was carried out using the avidin–biotin complex immunoperoxidase method. Samples were fixed in paraformaldehyde and embedded in paraffin. Sections were subjected to a pre-treatment step whereby they were immersed in 0.1% trypsin for 30 min at 37°C. Endogenous peroxidase activity was impeded with 1.5% hydrogen peroxide in methanol for 10 min at room temperature after which sections were blocked with a PBS solution containing either 5% goat serum for SULT2B1b or 5% horse serum (Vector Laboratories, Burlingame, California) for involucrin and filaggrin for 20 min at room temperature. Sections were incubated for 1 h with rabbit anti-SULT2B1b (1:500), mouse monoclonal anti-human involucrin (1:1) (YLEM, Rome, Italy), or mouse monoclonal anti-human filaggrin (1:200) (Biomedical Technologies, Stoughton, Massachusetts) in PBS followed by washing with PBS, incubation with either biotinylated antirabbit IgG (SULT2B1) or antimouse IgG (involucrin and filaggrin) for 20 min and stained using Vectastain ABC Kit (Vector Laboratories) according to the manufacturer's instructions. Staining was developed with diaminobenzidine (Vector laboratories), and after washing, slides were incubated in Myer's Hematoxylin (Muto Pure Chemicals, Tokyo, Japan).

Address correspondence to: Charles A. Strott, MD, Bldg 49, Rm 6A36, NIH, Bethesda, MD, 20892-4510, USA. Email: Chastro@mail.nih.gov

References

Ballabio A, Shapiro LJ: Steroid sulfatase deficiency and X-linked ichthyosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The Metabolic and Molecular Bases of Inherited Diseases. New York: McGraw-Hill, 1995; p 2999–3022

Bleau G, Bodley FH, Longpre J, Chapdelaine A, Roberts KD: Cholesterol sulfate I. Occurrence and possible function as an amphipathic lipid in the membrane of the human erythrocyte. Biochim Biophys Acta 352:1–9, 1974

Bleau G, Chapdelaine A, Roberts KD: The assay of cholesterol sulfate in biolgical material by enzmatic radioisotopic displacement. Can J Biochem 50:277–286, 1972

Bleau G, Lalumiere G, Chapdelaine A, Roberts KD: Red cell surface structure. Stabilization by cholesterol sulfate as evidenced by electron microscopy. Biochim Biophys Acta 375:220–223, 1975

Bouwstra JA, Dubbelaar FER, Gooris GS, Ponec M: The lipid organisation in the skin barrier. Acta Derm Venereol Suppl (Stockh) 208:23–30, 2000

Bouwstra JA, Gooris GS, Dubbelaar FER, Ponec M: Cholesterol sulfate and calcium affect stratum corneum lipid organization over a wide temperature range. J Lipid Res 40:2303–2312, 1999

Denning MF, Kazanietz MG, Blumberg PM, Yuspa SH: Cholesterol sulfate activates multiple protein kinase C isozymes and induces granular cell differentiation in cultured murine keratinocytes. Cell Growth Differ 6:1619–1626, 1995

Downing DT, Stewart ME, Wertz PW, Colton SW, Abraham W, Strauss JS: Skin lipids: An update. J Invest Dermatol 88:2s–6s, 1987

Elias PM, Williams ML, Maloney ME, Bonifas JA, Brown BE, Grayson S, Epstein EH Jr: Stratum corneum lipids in disorders of cornification. J Clin Invest 74:1414–1421, 1984

Falany CN: Enzymology of human cytosolic sulfotransferases. FASEB J 11:206–216, 1997

Falany CN, Wheeler J, Oh TS, Falany JL: Steroid sulfation by expressed human cytosolic sulfotransferases. J Steroid Biochem Mol Biol 48:369–375, 1994

Fuchs E: Epidermal differentiation: The bare essentials. J Cell Biol 111:2807–2814, 1990

Fuda H, Lee YC, Shimizu C, Javitt NB, Strott CA: Mutational analysis of human hydroxysteroid sulfotransferase SULT2B1 isoforms reveals that exon 1B

of the *SULT2B1* gene produces cholesterol sulfotransferase, whereas exon 1A yields pregnenolone sulfotransferase. J Biol Chem 277:36161–36166, 2002

Gray GM, Yardley HJ: Lipid compositions of cells isolated from pig, human, and rat epidermis. J Lipid Res 16:434–440, 1975

Hanley K, Wood L, Ng DC, *et al*: Cholesterol sulfate stimulates involucrin transcription in keratinocytes by increasing Fra-1, Fra-2, and Jun D. J Lipid Res 42:390–398, 2001

Her C, Wood TC, Eichler EE, Mohrenweiser HW, Ramagli IS, Siciliano MJ, Weinshilboum RM: Human hydroxysteroid sulfotransferase SULT2B1: two enzymes encoded by a single chromosome 19 gene. Genomics 53:284–295, 1998

Ishida-Yamamoto A, Takahashi H, Iizuka H: Immunoelectron microscopy links molecules and morphology in the studies of keratinization. Eur J Dermatol 10:429–435, 2000

Javitt NB, Lee YC, Shimizu C, Fuda H, Strott CA: Cholesterol and hydroxycholesterol sulfotransferases: Identification, distinction from dehydroepiandrosterone sulfotransferase, and differential tissue expression. Endocrinology 142:2978–2984, 2001

Jetten AM, George MA, Nervi C, Boone LR, Rearick JI: Increased cholesterolsulfate and cholesterol sulfotransferase activity in relation to the multistep process of differentiation in human epidermal keratinocytes. J Invest Dermatol 92:203–209, 1989

Kanitakis J, Ramirez-Bosca A, Reano A, Viac J, Roche P, Thivolet J: Filaggrin expression in normal and pathological skin. Virchows Archiv A Anat Histopathol 412:375–382, 1988

Kawabe S, Ikuta T, Ohba M, Chida K, Ueda Y, Yamanishi K, Kuroki T: Cholesterol sulfate Activates transcription of transglutaminase 1 gene in normal human keratinocytes. J Invest Dermatol 111:1098–1102, 1998

Lampe MA, Burlingame AL, Whitney JA, Williams ML, Brown BE, Roitman E, Elias PM: Human stratum corneum lipids: Characterization and regional variations. J Lipid Res 24:120–130, 1983

Lampe MA, Williams ML, Elias ER: Human epidermal lipids: Characterization and modulations during differentiation. J Lipid Res 24:131–140, 1983

Langlais J, Zollinger M, Plante L, Chapdelaine A, Bleau G, Roberts KD: Localization of cholesteryl sulfate in human spermatozoa in support of

a hypothesis for the mechanism of capacitation. Proc Natl Acad Sci USA 78:7266–7270, 1981

Long SA, Wertz PW, Strauss JS, Downing DT: Human stratum corneum polar lipids and desquamation. Arch Dermatol Res 277:284–287, 1985

Lyle S, Stanczak J, Ng K, Schwartz NB: Rat chondrosarcoma ATP-sulfurylase and adenosine 5′-phosphosulfate kinase reside on a single bifunctional protein. Biochemistry 33:5920–5925, 1994

Maloney ME, Williams ML, Epstein EH, Michael Y, Law L, Fritsch PO, Elias PM: Lipids in the pathogenesis of ichthyosis: Topical cholesterol sulfate-induced scaling in hairless mice. J Invest Dermatol 83:252–256, 1984

Nagata K, Yamazoe Y: Pharmacogenetics of sulfotransferase. Annu Rev Pharmacol Toxicol 40:159–176, 2000

Nemes Z, Steinert PM: Bricks and mortar of the epidermal barrier. Exp Mol Med 31:5–19, 1999

Radominska A, Comer KA, Zimniak P, Falany J, Iscan M, Falany CN: Human liver steroid sulphotransferase sulphates bile acids. Biochem J 272:597–604, 1990

Roberts KD: Sterol sulfates in the epididymis; synthesis and possible function in the reproductive process. J Biol Chem 337–341, 1987

Roop D: Defects in the barrier. Science 267:474–475, 1995

Sato J, Denda M, Nakanishi J, Nomura J, Koyama J: Cholesterol sulfate inhibits proteases that are involved in desquamation of stratum corneum. J Invest Dermatol 111:189–193, 1998

Shimizu C, Fuda H, Yanai H, Strott CA: Conservation of the hydroxysteroid sulfotransferase SULT2B1 gene structure in the mouse: Pre- and postnatal expression, kinetic analysis of isoforms, and comparison with prototypical SULT2A1. Endocrinology 144:1186–1193, 2003

Strott CA, Higashi Y: Cholesterol sulfate in human physiology: What's it all about? J Lipid Res 44:1–11, 2003

Vaitukaitis JL: Production of antisera with small doses of immunogen: Multiple intradermal injections. Methods Enzymol 73:46–52, 1981

Williams ML, Elias PM: Stratum corneum lipids in disorders of cornification. J Clin Invest 68:1404–1410, 1981

Williams ML, Hughes-Fulford M, Elias PM: Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and sterol synthesis by cholesterol sulfate in cultured fibroblasts. Biochim Biophys Acta 845:349–357, 1985